$CD4^+CD25^+$ regulatory T cells attenuate cisplatin-induced nephrotoxicity in mice

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Nephrotoxicity limits the use of cisplatin, a widely used chemotherapeutic agent for treatment of various malignancies. Overall, $CD4^+$ T cells mediate cisplatin-induced renal injury; however, the $CD4^+CD25^+$ regulatory T-cell subset ($CD4^+CD25^+$ Treg) has broad suppressive effects on many different cell types. In this study, we determined whether $CD4^+CD25^+$ Treg cells had protective effects against cisplatin-induced acute renal injury in *nu/nu* mice that lack mature T cells. In these mice, there was marked attenuation of the decreased survival, renal dysfunction and tubular injury, renal tumor necrosis factor-α, and interleukin-1β cytokine levels. Furthermore, renal macrophage accumulation was reduced in $CD4^+CD25^+$ Treg cell-adoptive transferred *nu/nu* mice compared with control mice. Infusion of $CD4^+CD25^+$ Treg cells into wild-type Balb/c mice reduced serum blood urea nitrogen and creatinine levels equivalent to those in *nu/nu* mice and extended their survival time after cisplatin injection. In contrast, depletion of $CD4^+CD25^+$ Treg cells in wild-type mice exacerbated kidney injury after cisplatin administration. Transcription factor *Foxp3*-positive cells (Treg cells) were detected in the kidneys of *nu/nu* mice after cisplatin injection. Our results suggest that $CD4^+CD25^+$ Treg cells directly affect cisplatin nephrotoxicity and their modulation represents an additional treatment strategy.

Kidney International (2010) **78,** 1100–1109; doi:10.1038/ki.2010.139; published online 12 May 2010

KEYWORDS: cisplatin; nephrotoxicity; regulatory T cell

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Received 6 August 2009; revised 24 February 2010; accepted 16 March 2010; published online 12 May 2010

Cisplatin is widely used as a chemotherapeutic agent in various cancers, including ovarian, head and neck, and germ cell tumors. However, nephrotoxicity is commonly reported as an adverse effect of cisplatin.[1] Indeed, approximately 25–30% of patients experience renal dysfunction after a single dose of cisplatin.[2] Therefore, it is important to understand the pathophysiological mechanism responsible for cisplatin nephrotoxicity for the development of adjunctive therapies to reduce this side effect.

Recent studies in experimental models have confirmed that T cells have a pathophysiological role in cisplatin nephrotoxicity. T cell-deficient *nu/nu* mice show less kidney injury and tumor necrosis factor (TNF)-α production after cisplatin administration when compared with normal mice. Furthermore, CD4-deficient mice have been found to have a marked degree of protection from renal dysfunction, which indicates that $CD4^+$ T cells are the primary T-cell subsets that mediate cisplatin-induced renal injury.[3] However, a study using a chimeric model conducted by Reeves and colleagues[4] demonstrated that the production of TNF-α, which is a key cytokine involved in the inflammatory response during cisplatin nephrotoxicity, is more closely related to resident kidney cells than bone marrow-derived immune cells. These two studies demonstrate the complexity of the mechanism that is potentially important in cisplatin-induced acute kidney injury well.[5] Therefore, it is necessary to identify a specific target to reduce the toxicity associated with cisplatin, which will require a novel attempt to suppress various immune responses by different cell types.

Recently, $CD4^+CD25^+$ regulatory T ($CD4^+CD25^+$ Treg) cells have been shown to have a pivotal role in the maintenance of tolerance in the immune system.[6–8] There is a great deal of convincing evidence that $CD4^+CD25^+$ Treg cells can suppress the development of autoimmune diseases, such as rheumatoid arthritis,[9] multiple sclerosis,[10] and lupus.[11] Besides having a role in autoimmune diseases, $CD4^+CD25^+$ Treg cells have regulatory function in the control of transplantation tolerance,[12] tumor immunity,[13] allergy,[14] and infection.[15] These broad suppressive effects in various studies indicate that $CD4^+CD25^+$ Treg cells can inhibit the function of many different cell types.

Currently available evidence indicates that $CD4^+CD25^+$ Treg cells can suppress $CD4^+$ T cell-mediated pathology in the kidneys. For example, it has been shown that $CD4^+CD25^+$ Treg cells inhibit antiglomerular basement membrane glomerulonephritis, which is a complement-dependent Th1-predominant mouse model.[16] In addition, the ability of $CD4^+CD25^+$ Treg cells to inhibit the innate immune response has been thoroughly investigated.[17] It is known that macrophages, which are an important component of innate immunity, are strongly related to the pathogenesis of various renal diseases. By migrating to the kidney, macrophages can contribute to tissue damage and their production of proinflammatory cytokines eventually progresses to renal failure.[18] Recent studies have suggested that $CD4^+CD25^+$ Treg cells can modulate macrophages by inhibiting their activation, leading to reduced glomerular and interstitial injury in adriamycin nephropathy.[19]

In this study, we evaluated $CD4^+CD25^+$ Treg cells to determine whether they could attenuate cisplatin-induced nephrotoxicity by suppressing their effect on innate immune reaction. To examine the effects of $CD4^+CD25^+$ Treg cells, we used the $CD4^+CD25^+$ Treg-cell adoptive transfer or depletion methods in T cell-deficient nude mice (*nu/nu*) and wild-type (WT) Balb/c mice and evaluated survival, kidney function, histology, and cytokine expression after cisplatin administration. In addition, we sought to examine the migration of *Foxp3*-positive cells into the kidney after cisplatin injection.

RESULTS

Adoptive transfer of $CD4^+CD25^+$ Treg cells improved the survival of *nu/nu* mice

All mice received a single dose of cisplatin at 40 mg/kg and were then evaluated for 96 h. At 72 h after cisplatin injection, all Balb/c mice were dead, whereas 15 of the 16 *nu/nu* mice were still alive (Figure 1a). The reduced early mortality in *nu/nu* mice observed in this study was similar to the results of a study conducted by Liu *et al.*[3] found in 2006. On the basis of this reproductive result, the next series of studies were conducted. However, *nu/nu* mice also started to die as more time passed. In particular, 9 out of 16 *nu/nu* mice that received $CD4^+CD25^+$ Treg cells were alive at 96 h after the injection of cisplatin, whereas only 3 out of 16 $CD4^+CD25^-$ T cell-reconstituted *nu/nu* mice survived (Figure 1b).

$CD4^+CD25^+$ Treg cells attenuated renal dysfunction of cisplatin-treated mice

Cisplatin causes acute renal injury, which induces an increase in serum creatinine and blood urea nitrogen (BUN). Therefore, kidney function was monitored by measuring the serum creatinine and BUN levels after intraperitoneal injection of saline or cisplatin. Cisplatin administration led to the development of acute kidney injury with an increase in creatinine (saline 0.28 ± 0.05 mg/dl; cisplatin 2.4 ± 0.48 mg/dl) and BUN (saline 29 ± 1.8 mg/dl; cisplatin 185 ± 22 mg/dl) levels being observed within 24 h of cisplatin administration

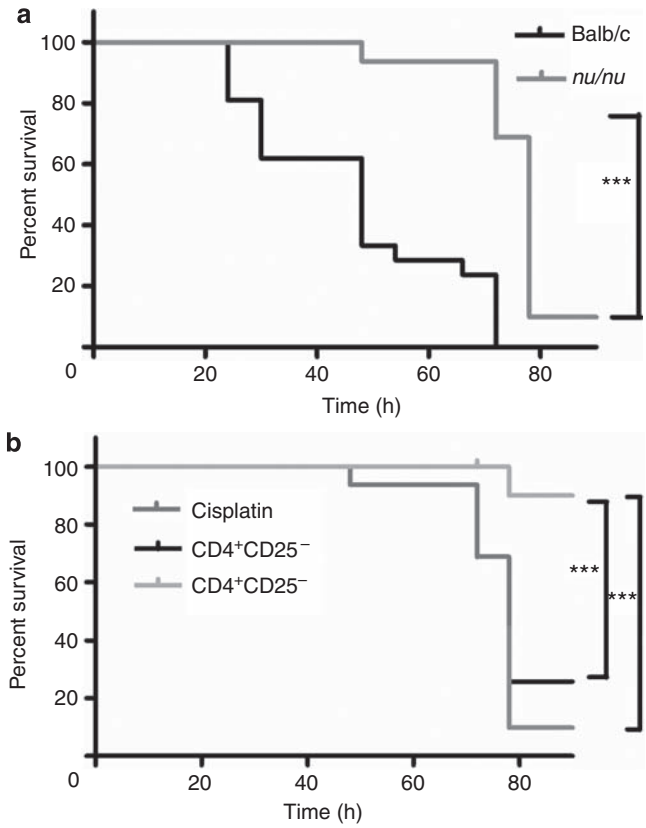

Figure 1 | Survival in cisplatin-treated mice. All mice received a single dose of cisplatin intraperitoneally (40 mg/kg body weight) and were followed up for up to 96 h. (**a**) *nu/nu* Mice had a 69% survival rate, whereas all Balb/c mice expired (***$P < 0.001$ vs Balb/c; $n = 16$ to 21). (**b**) *nu/nu* Mice were reconstituted by tail intravenous injection with 1×10^6 $CD4^+CD25^-$ T cells or $CD4^+CD25^+$ Treg cells 10 days before cisplatin administration. $CD4^+CD25^+$ Treg cell-reconstituted mice had a longer survival time than did the other mice (***$P < 0.001$ vs cisplatin; $n = 16$). Treg, T regulatory cell.

in WT mice. In contrast, *nu/nu* mice that received cisplatin showed significant attenuation of the increase in creatinine (0.3 ± 0.04 mg/dl) and BUN (38 ± 4.4 mg/dl) levels when compared with WT mice. There were no differences among *nu/nu* mice groups at 24 h; however, 48 h after the administration of cisplatin, increases in the serum creatinine and BUN levels were observed in *nu/nu* mice and $CD4^+CD25^-$ T cell-adoptive transferred *nu/nu* mice. Furthermore, significant attenuation of cisplatin-medicated renal dysfunction was observed in $CD4^+CD25^+$ Treg cell-adoptive transferred *nu/nu* mice (Figure 2). However, the transfer of $CD4^+CD25^-$ T cell into *nu/nu* mice could not exacerbate renal dysfunction, which contradicts the earlier observations made by Rabb and co-workers.[3] In particular, the studies conducted by Rabb *et al.* injected approximately 3×10^6 T cells into *nu/nu* mice 3 weeks before cisplatin administration, whereas we transferred $\sim 1 \times 10^6$ $CD4^+CD25^-$ T cells into *nu/nu* mice 10 days before cisplatin injection. The composition and number of T cells reported by Rabb *et al.* were different from those observed in this study. Therefore, we cannot exclude the possibility that other T-cell

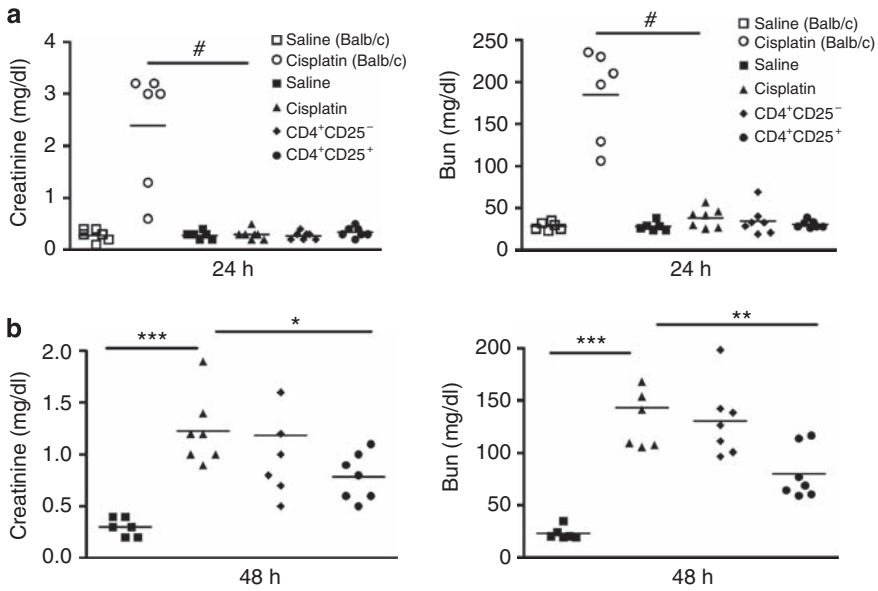

Figure 2 | Effect of CD4$^+$CD25$^+$ Treg cells on renal dysfunction in mice. *nu/nu* Mice were reconstituted by tail intravenous injection with 1×10^6 CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ T cells 10 days before cisplatin administration. Balb/c mice: saline treatment alone (saline (Balb/c)); cisplatin treatment alone (cisplatin (Balb/c)). *nu/nu* Mice: saline treatment alone (saline); cisplatin treatment alone (cisplatin); transferred CD4$^+$CD25$^-$ T-cell and cisplatin treatment (CD4$^+$CD25$^-$); transferred CD4$^+$CD25$^+$ Treg cell and cisplatin treatment (CD4$^+$CD25$^+$). Creatinine and BUN levels were measured at 24 and 48 h, respectively, after saline or cisplatin injection. Changes in creatinine (creatinine 48 h: saline 0.30 ± 0.04 mg/dl; cisplatin 1.2 ± 0.13 mg/dl; CD4$^+$CD25$^+$ 0.79 ± 0.09 mg/dl) and BUN (BUN 48 h: saline 23 ± 2.4 mg/dl; cisplatin 140 ± 15 mg/dl; CD4$^+$CD25$^+$ 80 ± 9.4 mg/dl) levels were measured (*$P<0.05$, **$P<0.01$, ***$P<0.001$, $^{\#}P<0.001$ vs cisplatin, two-tailed paired *t*-test; $n=6$ to 7). BUN, blood urea nitrogen; Treg, T regulatory cell.

subsets have a synergistic pathological effect with CD4$^+$CD25$^-$ T cells on cisplatin-induced nephrotoxicity or that the number of transferred CD4$^+$CD25$^-$ T cells and time allowed for expansion in the lymphoid organs are not sufficient to reveal the pathological effects of CD4$^+$CD25$^-$ T cells.

Adoptive transfer of CD4$^+$CD25$^+$ Treg cells reduced renal injury in *nu/nu* mice

The degree of renal tubular injury in saline, CD4$^+$CD25$^-$ T, and CD4$^+$CD25$^+$ Treg cell-treated groups was observed at 48 h after cisplatin administration. *nu/nu* Mice that received CD4$^+$CD25$^+$ Treg cells had less renal injury than did *nu/nu* mice that received saline. However, *nu/nu* mice that received CD4$^+$CD25$^-$ T cells and *nu/nu* mice showed extensive renal injury at 48 h after cisplatin injection (Figure 3).

Adoptive transfer of CD4$^+$CD25$^+$ Treg cells reduced macrophage infiltration in the kidneys of mice

Macrophages were found in the interstitium of kidney sections in the cisplatin group of mice and the CD4$^+$CD25$^-$ group of mice. At 48 h after cisplatin administration, more F4/80-positive macrophages were observed in the cisplatin group of mice and the CD4$^+$CD25$^-$ group of mice. Conversely, F4/80-positive macrophages were rarely detected in the CD4$^+$CD25$^+$ group of mice (Figure 4).

Effect of CD4$^+$CD25$^+$ T cells on proinflammatory cytokines in cisplatin nephrotoxicity

To investigate proinflammatory molecules generated by cisplatin renal injury, cytokine levels of TNF-α and interleukin (IL)-1β were measured at 48 h after cisplatin administration. Cisplatin-treated mice showed increased levels of TNF-α and IL-1β at 48 h after cisplatin injection. However, *nu/nu* mice that received CD4$^+$CD25$^+$ Treg cells showed reduced TNF-α (saline 2200 ± 180 pg/mg; cisplatin 4100 ± 290 pg/mg; CD4$^+$CD25$^-$ 4531 ± 526.5 pg/mg; CD4$^+$CD25$^+$ 3181 ± 85.20 pg/mg) and IL-1β (saline 1200 ± 160 pg/mg; cisplatin 3972 ± 363.8 pg/mg; CD4$^+$CD25$^-$ 3668 ± 515.5 pg/mg; CD4$^+$CD25$^+$ 1969 ± 63.82 pg/mg) levels (Figure 5).

Effect of infused CD4$^+$CD25$^+$ Treg cells on cisplatin nephrotoxicity in Balb/c mice

After investigating the protective effects of CD4$^+$CD25$^+$ Treg cells on cisplatin nephrotoxicity in *nu/nu* mice, we tested whether infused CD4$^+$CD25$^+$ Treg cells might have had a protective role in cisplatin-induced renal injury. Approximately 3×10^6 CD4$^+$CD25$^+$ Treg cells were injected into Balb/c mice through the tail vein. At 10 days after injection of the CD4$^+$CD25$^+$ Treg cells, mice received a single dose of cisplatin at a concentration of 25 mg/kg. Interestingly, mice infused with CD4$^+$CD25$^+$ Treg cells showed prolonged survival times and attenuations of the serum creatinine and BUN levels (Figure 6).

Effect of CD4$^+$CD25$^+$ Treg cell depletion on cisplatin nephrotoxicity in Balb/c mice

A dose of 0.25 mg of anti-CD25 antibody was injected daily for 3 days before cisplatin administration. All Balb/c mice received a single dose of cisplatin at 25 mg/kg. There were no

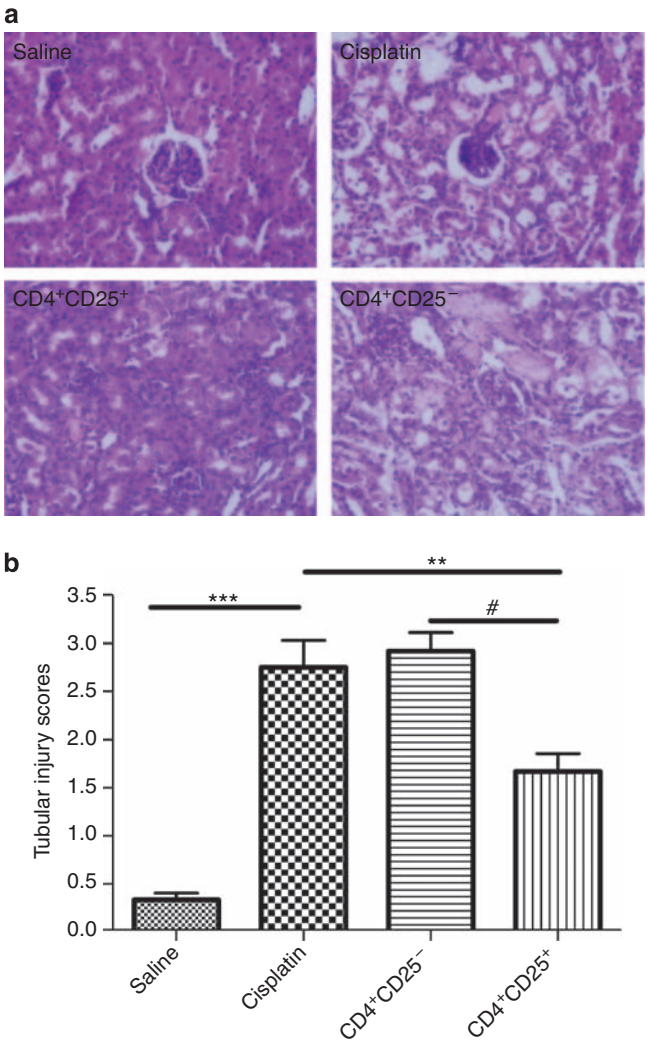

Figure 3 | Effect of CD4$^+$CD25$^+$ Treg cells on renal histology in mice. Sections of kidney were stained with H&E 48 h after cisplatin injection. (**a**) Saline treatment alone (saline); cisplatin treatment alone (cisplatin); transferred CD4$^+$CD25$^-$ T-cell and cisplatin treatment (CD4$^+$CD25$^-$); transferred CD4$^+$CD25$^+$ Treg cell and cisplatin treatment (CD4$^+$CD25$^+$). (**b**) Tubular injury was scored using the semi-quantitative evaluation method. The CD4$^+$CD25$^+$ Treg cell-injected mice had significantly less tubular injury than did CD4$^+$CD25$^-$ T cell-injected mice and cisplatin-treated control mice (**$P<0.01$, ***$P<0.001$ vs cisplatin, $^{\#}P<0.001$ vs CD4$^+$CD25$^+$, two-tailed paired *t*-test; $n=4$). H&E, hematoxylin and eosin; Treg, T regulatory cell.

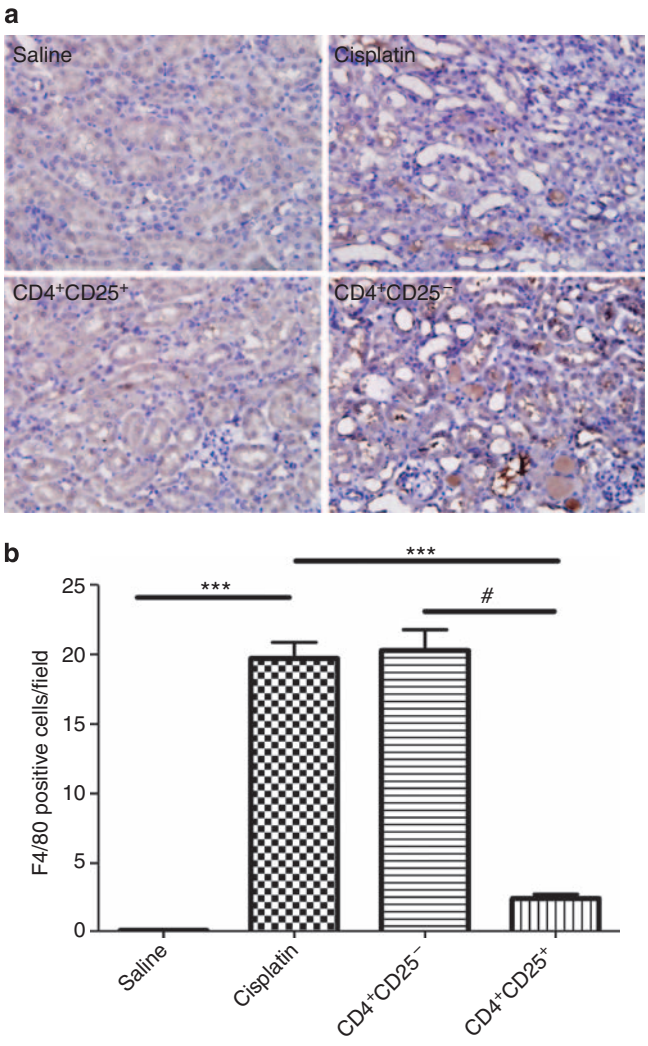

Figure 4 | Effect of CD4$^+$CD25$^+$ Treg cells on macrophage infiltration in the kidneys of mice. (**a**) Accumulation of macrophages in the kidneys was detected by immunostaining of F4/80-positive cells at 48 h after cisplatin administration. Saline treatment alone (saline); cisplatin treatment alone (cisplatin); transferred CD4$^+$CD25$^-$ T-cell and cisplatin treatment (CD4$^+$CD25$^-$); transferred CD4$^+$CD25$^+$ Treg cell and cisplatin treatment (CD4$^+$CD25$^+$). (**b**) The numbers of F4/80-positive cells in each section were counted in 10 fields per slide at an original magnification of $\times 400$. Macrophage infiltration was significantly reduced in CD4$^+$CD25$^+$ Treg cell-reconstituted mice compared with CD4$^+$CD25$^-$ T cell-injected mice and cisplatin-treated control mice (**$P<0.01$, ***$P<0.001$ vs cisplatin, $^{\#}P<0.001$ vs CD4$^+$CD25$^+$, two-tailed paired *t*-test; $n=4$). Treg, T regulatory cell.

differences among groups at 24 and 48 h; however, 72 h after cisplatin injection, increases in the serum creatinine and BUN levels were observed in CD4$^+$CD25$^+$ Treg cell-depleted mice (Figure 7b). Furthermore, exacerbation of renal injury was observed in CD4$^+$CD25$^+$ Treg cell-depleted mice at 72 h after cisplatin treatment when compared with normal Balb/c mice (Figure 7c). CD4$^+$CD25$^+$ Treg cell-depleted mice also died slightly earlier than did normal Balb/c mice (Figure 7d), indicating that CD4$^+$CD25$^+$ Treg cells have a functional role in cisplatin nephrotoxicity.

Assessment of CD4$^+$CD25$^+$ Treg-cell migration into the kidney upon cisplatin treatment

For assessment of CD4$^+$CD25$^+$ Treg migration into the kidney, we used *Foxp3*^{EGFP} mice initially described by Haribhai *et al.*[20] These mice were derived with a bicistronic *Foxp3* locus that coexpresses the enhanced green fluorescence protein (EGFP) under control of the endogenous *Foxp3* promoter, so that the expression of *Foxp3*, a key transcriptional factor in CD4$^+$CD25$^+$ Treg cell, could be analyzed in real time using the EGFP fluorescence signal. To visualize and

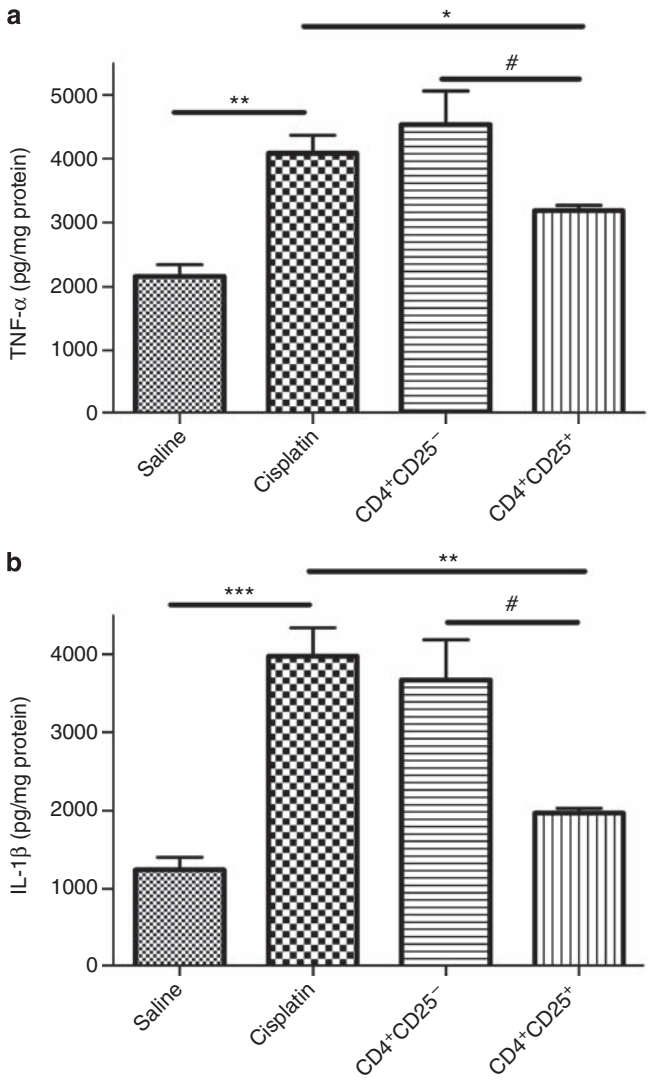

Figure 5 | Proinflammatory cytokines in the kidney. Proinflammatory cytokines in the kidney were measured by ELISA at 48 h after cisplatin injection. When compared with cisplatin-treated control mice, the elevation of TNF-α (**a**; saline 2200 ± 180; cisplatin 4100 ± 290; $CD4^+CD25^-$ 4531 ± 526.5; $CD4^+CD25^+$ 3181 ± 85.20) and IL-1β (**b**; saline 1200 ± 160; cisplatin 4000 ± 360; $CD4^+CD25^-$ 3668 ± 515.5; $CD4^+CD25^+$ 1969 ± 63.82) was significantly lower in $CD4^+CD25^+$ Treg cell-reconstituted mice. (**$P<0.01$, ***$P<0.001$ vs cisplatin, $^{\#}P<0.001$ vs $CD4^+CD25^+$, two-tailed paired *t*-test; $n=4$). ELISA, enzyme-linked immunosorbent assay; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; Treg, T regulatory cell.

quantify the degree of Treg migration, confocal microscopy was used with kidney samples obtained from $CD4^+CD25^+$ Treg-transferred *nu/nu* mice that were killed at various time points after cisplatin injection. Our results showed that Foxp3-positive cells migrated directly to the kidney in mice at an early time point (Figure 8).

DISCUSSION

In this study, we provide strong evidence that $CD4^+CD25^+$ Treg cells effectively attenuate the adverse effects of cisplatin nephrotoxicity. Our data showed that the transfer of $CD4^+CD25^+$ Treg cells into *nu/nu* mice significantly extended their survival time and reduced renal dysfunction in a cisplatin-induced renal nephrotoxicity model. In addition, infiltration of macrophages into the kidney deceased. Furthermore, renal injury was prevented in $CD4^+CD25^+$ Treg cell-transferred mice. After cisplatin injection, the levels of proinflammatory cytokine, TNF-α and IL-1β were increased in all mice, whereas these increases were attenuated in $CD4^+CD25^+$ Treg cell-transferred mice.

An inflammatory basis for cisplatin toxicity was shown with a role for intercellular adhesion molecule-1, TNF-α, and other proinflammatory molecules.[21] Many studies have suggested that TNF-α has a key role in cisplatin-induced nephrotoxicity.[21,22] However, there are various cells that can produce TNF-α in the kidney, which makes understanding the mechanism responsible for cisplatin nephrotoxicity difficult. Reeves and colleagues[4] demonstrated that TNF-α is produced by resident kidney cells rather than bone marrow-derived immune cells. Conversely, recent work by Rabb *et al.* demonstrated that T cell-deficient *nu/nu* mice have attenuated cisplatin renal injury when compared with WT mice. Furthermore, reconstitution of T cells into T cell-deficient *nu/nu* mice leads to increased renal dysfunction and TNF-α production in a mouse model, which indicates that infiltrating $CD4^+$ T cells have a significant role in the pathogenesis in cisplatin-induced nephrotoxicity.[3] Our 96-h follow-up survival data showed that *nu/nu* mice started to die after cisplatin injection, although T cell-deficient *nu/nu* mice had a longer survival time than did WT Balb/c mice. These results indicate that infiltration of T cells is not the only pathogenic factor in cisplatin-induced nephrotoxicity.

Macrophages have been proposed as important mediators of injury in most types of primary and secondary human kidney diseases.[23] The degree of macrophage infiltration is a major parameter used to predict the progression of disease and the activation of macrophage expressed inflammatory mediators, including nitric oxide and TNF-α, which are major pathogenic factors associated with cisplatin nephrotoxicity. Indeed, a clear association has been shown between macrophage accumulation and the severity of renal injury.[18] In addition, various reports have shown that depletion of macrophages reduced renal injury.[24,25]

The suppressive effect of $CD4^+CD25^+$ Treg cells on $CD4^+CD25^-$ T cell has been well documented. However, the protection effect of $CD4^+CD25^+$ Treg cells in *nu/nu* mice after cisplatin administration showed that $CD4^+CD25^+$ Treg cells have a more general immunosuppressive effect in our mice model. Maloy *et al.*[26] reported that the presence of Tregs resulted in reduced recruitment of monocyte/macrophage to the site of inflammation. In addition, a recent study has shown that when monocytes were cocultured with $CD4^+CD25^+$ Treg cells, the expression of activating markers of monocytes such as CD40, CD80, and HLA II were significantly lower when compared with monocytes that were cultured in the presence of $CD4^+CD25^-$ T cells. Moreover,

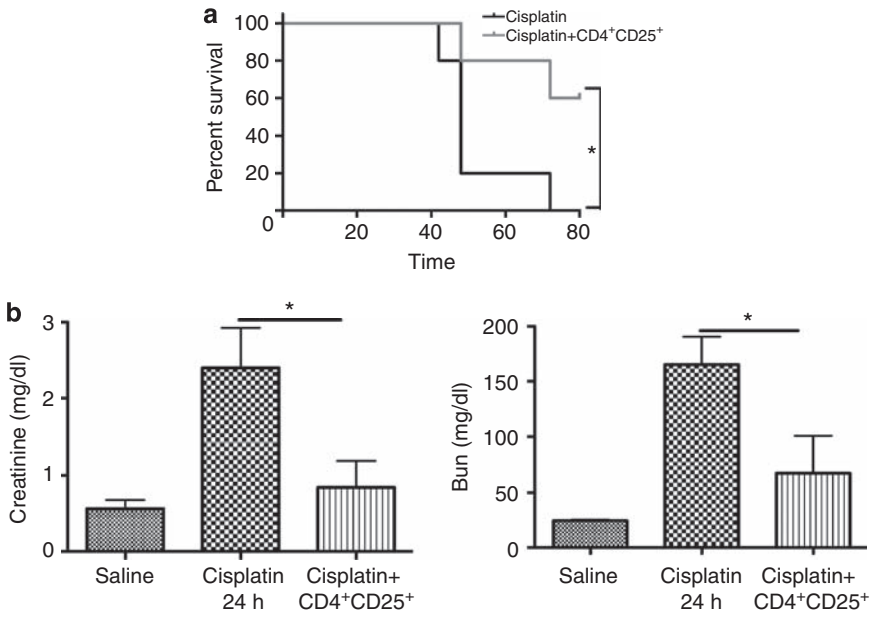

Figure 6 | Effect of infused $CD4^+CD25^+$ Treg cells on cisplatin nephrotoxicity in Balb/c mice. Balb/c mice were reconstituted by intravenous injection with 3×10^6 $CD4^+CD25^+$ T cells 10 days before cisplatin administration. All mice received a single dose of cisplatin or saline intraperitoneally (25 mg/kg body weight). (**a**) Survival rate and (**b**) BUN and serum creatinine (**b**) were measured 24 h after cisplatin administration. (*$P < 0.05$ vs cisplatin; $n = 5$). BUN, blood urea nitrogen; Treg, T regulatory cell.

$CD4^+CD25^+$ Treg cells affected the monocyte/macrophage cytokine profile in response to strong proinflammatory stimuli, such as lipopolysaccharides.[27] Similarly, in a murine model of burn injury, there was evidence of $CD4^+CD25^+$ Treg cells suppressing innate immune activation by controlling TLR (Toll-like receptor)4 and TLR2 responses.[17]

The results of this study show that the therapeutic efficacy of $CD4^+CD25^+$ Treg cells in murine cisplatin-induced nephrotoxicity occurs by suppression of the innate immune pathology. In particular, renal damage and the infiltration of macrophages in response to cisplatin were significantly attenuated by the transfer of $CD4^+CD25^+$ Treg cells. The importance of TNF-α in the pathogenesis of cisplatin-induced renal dysfunction has been well documented.[21,28] Although it is known that TNF-α is mainly produced by resident kidney cells, reduction of the TNF-α level in $CD4^+CD25^+$ Treg-transferred mice clearly indicated that $CD4^+CD25^+$ Treg cells could affect renal TNF-α production. A recent report has shown that activation of TLR4 present on renal parenchymal cells triggers an innate immune response that mediates cisplatin-induced acute renal failure.[5] The results presented in a previous study fit with our researches that mouse $CD4^+CD25^+$ Treg cells suppressed innate immune pathology.

On the basis of the reduction of renal damage after $CD4^+CD25^+$ Treg transfer, we assumed that Tregs migrate to the kidney after cisplatin injection. $CD4^+CD25^+$ Treg cells arise in the thymus, represent 5–10% of $CD4^+$ T cells in the periphery, and are distinguished by the expression of transcription factor *Foxp3*.[29,30] Therefore, we sought to examine the migration of *Foxp3*-positive cells into the kidney after cisplatin injection. Our results showed that *Foxp3*-positive cells infiltrated the kidney at an early time point in *nu/nu* mice. T-cell trafficking into a target organ is an important factor for T cell-mediated injury in kidney diseases. Studies in experimental models have confirmed that T cells significantly increased in the kidneys of normal mice after cisplatin administration at 1 h, peaked at 12 h, and declined by 24 h.[3] In this study, $CD4^+CD25^+$ Treg cells migrated to the kidney at an early time point in *nu/nu* mice. This indicated that $CD4^+CD25^+$ Treg cells could have a direct interaction with cisplatin-induced renal inflammation.

Considering the protective effect of renal injury by transferred $CD4^+CD25^+$ Treg cells in *nu/nu* mice, one could assume that manipulating $CD4^+CD25^+$ Treg cells could modulate the harmful effects of cisplatin in WT mice. Thus, we transferred $CD4^+CD25^+$ Treg cells into WT mice or depleted $CD4^+CD25^+$ Treg cells in WT mice. We found that the transfer of $CD4^+CD25^+$ Treg cells into WT mice reduced the adverse effect of cisplatin, whereas depletion of $CD4^+CD25^+$ Treg cells exacerbated kidney injury. These findings confirm that $CD4^+CD25^+$ Treg cells have a dynamic role in the regulation of cisplatin-induced nephrotoxicity.

In summary, in this study, we show for the first time the protective effect of $CD4^+CD25^+$ Treg cells on cisplatin-induced nephrotoxicity in mice. This study indicates that $CD4^+CD25^+$ Treg cells migrate to the kidney and are directly related to the regulation of cisplatin nephrotoxicity. Therefore, we believe that understanding $CD4^+CD25^+$ Treg cell function in cisplatin nephrotoxicity might represent an additional treatment strategy for cisplatin-induced nephrotoxicity.

MATERIALS AND METHODS

Mice

Nu/nu male mice (C. Cg-*Foxnl*-nu/ CrljBg) and their Balb/c male littermates (6 to 8 weeks of age, weighing 20–25 g) were purchased from Orient Bio (Seungnam, South Korea). *Foxp3*^{EGFP} Balb/c (C. Cg-*Foxp3*^{tm2Tch}/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were kept under pathogen-free conditions with air conditioning and a 12-h light/dark cycle. In addition, all mice had free access to food and water during the experiments. The study was approved by the University of Kyung Hee Animal Care and Use Committee.

Cell isolation and T-cell reconstitution

$CD4^+CD25^+$ Treg and $CD4^+CD25^-$ T cells were isolated from the spleens obtained from male Balb/c mice using magnetic bead separation ($CD4^+CD25^+$ regulatory T-cell kit; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, non-$CD4^+$ T cells were depleted using biotinylated antibody cocktail and anti-biotin microbeads. $CD4^+CD25^-$ T cells were positively selected from $CD4^+CD25^+$ Treg cells using PE (phycoerythrin)-labeled anti-CD25 mAb and anti-PE microbeads. The purity of both populations was controlled by flow cytometry analysis and routinely reached >90%. *nu/nu* Mice (1×10^6 $CD4^+CD25^-$ T cells or $CD4^+CD25^+$ Treg cells) Balb/c mice (3×10^6 $CD4^+CD25^+$ Treg cells) were reconstituted by tail intravenous injection 10 days before cisplatin administration.

Induction of acute nephrotoxicity

Cisplatin (*cis*-diammineplatinum II dichloride; Sigma-Aldrich, St Louis, MO, USA) was dissolved in 0.9% saline at a concentration of 1 mg/ml. Mice were administered a single intraperitoneal injection of either cisplatin (40 or 25 mg/kg mice body weight) or an equal volume of saline. Mice were killed at 48 (*nu/nu* mice) or 72 h (Balb/c mice) after the administration of cisplatin for histology and kidney cytokine array analysis. All collected mouse kidneys were either fixed in 4% paraformaldehyde for histology/immunohistochemistry or snap-frozen with liquid nitrogen for tissue cytokine array.

Assessment of renal function and cytokines

Renal function was assessed by measurements of BUN and serum creatinine using FUJI DRI-CHEM 3500i (Fuji Photo Film, Tokyo, Japan). To examine the proinflammatory

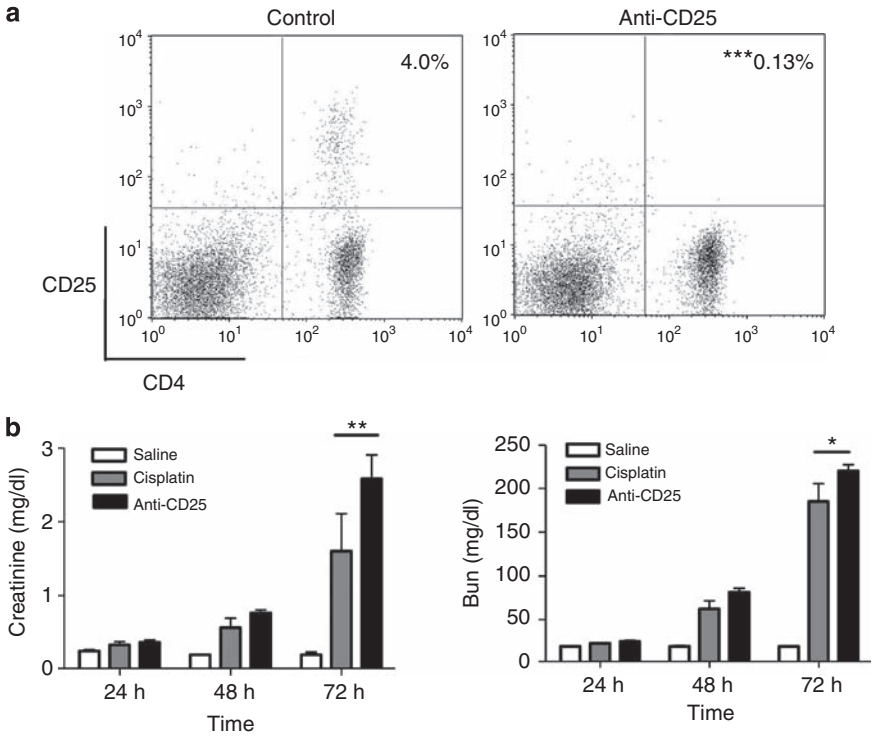

Figure 7 | Effect of $CD4^+CD25^+$ Treg cells depletion on cisplatin nephrotoxicity in Balb/c mice. Saline treatment alone (saline); cisplatin treatment alone (cisplatin); anti-CD25 antibody and cisplatin treatment (anti-CD25). (**a**) Balb/c mice received daily injections of 0.25 mg of anti-CD25 antibody for 3 days before cisplatin administration. The efficacy of $CD4^+CD25^+$ Treg cells depletion was confirmed by flow cytometry analysis using PE-anti-mouse CD25 and FITC-anti-mouse CD4 (***$P<0.001$ vs control, $n=4$). (**b**) Creatinine and BUN levels were measured at 24, 48, and 72 h after saline or cisplatin injection. Changes in creatinine and BUN levels were measured (*$P<0.05$, **$P<0.01$ vs cisplatin, $n=6$). (**c**) Sections of kidney were stained with H&E 72 h after cisplatin injection. Tubular injury was scored using the semi-quantitative evaluation method (*$P<0.05$ vs cisplatin, $n=6$). (**d**) Survival data between $CD4^+CD25^+$ Treg cells depletion mice and wild-type Balb/c mice ($n=11$). BUN, blood urea nitrogen; FITC, fluorescein isothiocyanate; H&E, hematoxylin and eosin; PE, phycoerythrin; Treg, T regulatory cell.

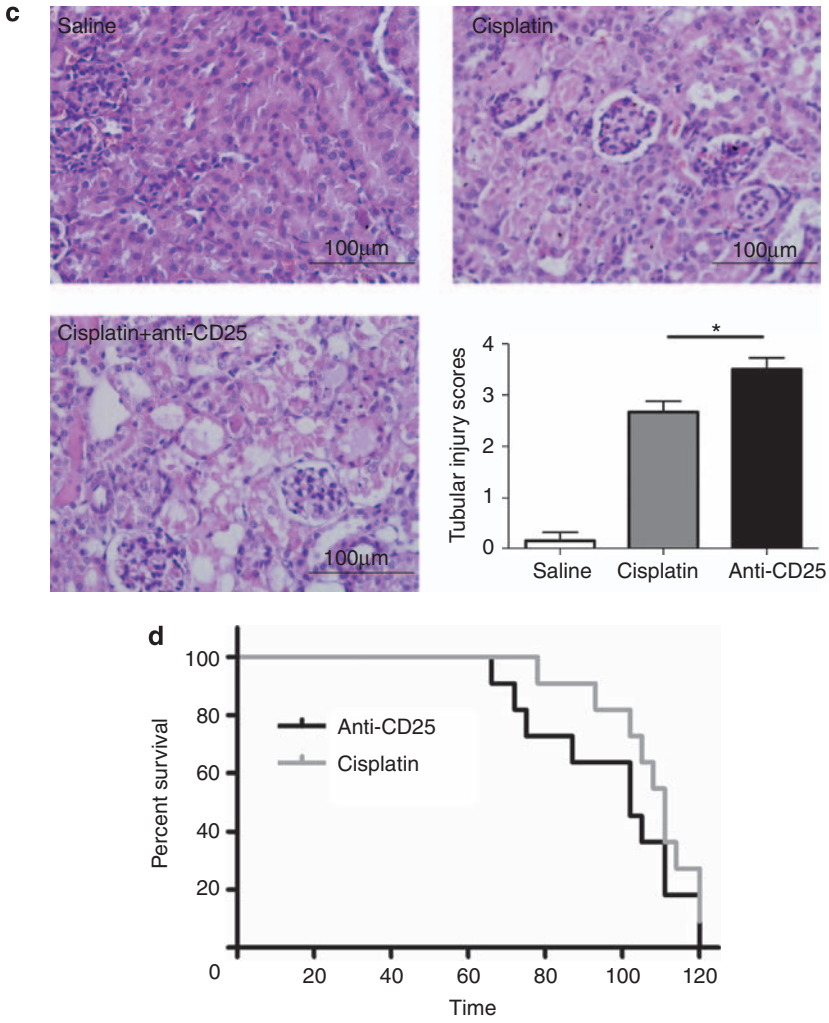

Figure 7 | Continued.

cytokines after cisplatin administration, protein levels of TNF-α and IL-1β were measured in the kidneys using enzyme-linked immunosorbent assay (ELISA; BD Biosciences, San Diego, CA, USA). Briefly, snap-frozen kidney tissue was homogenized in a PRO-PREP protein extraction solution (iNtRON Biotechnology, Seongnam, Korea), and the supernatant was used for kidney proinflammatory cytokine array. The protein concentrations in the supernatants were determined using a BCATH Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The protein levels of cytokines were corrected for the total amount of protein, and the results were expressed as pg/mg.

Histological examination

Kidney tissue was fixed in 4% paraformaldehyde and then embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin. Three pathologists who were blinded to the experiments scored the degree of tubular injury. Renal tubular injury was assessed using a semi-quantitative score, in which the percentage of cortical tubules showing epithelial necrosis was assigned a score of 0, none; 1, $<10\%$; 2, 10–25%; 3, 25–75%; 4, $>75\%$.

Immunohistochemical staining of macrophage

To evaluate macrophage infiltration into the kidneys after cisplatin administration, immunohistochemical staining for macrophages was performed on paraffin-embedded kidney tissue. Briefly, after deparaffinization and rehydration, kidney sections were pressure cooked in an antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA) for 3 min and then immersed in 3% hydrogen peroxide methanol for 30 min to quench the endogenous peroxidase. After treatment with blocking buffer, the slides were incubated overnight at 4°C with a rat anti-mouse F4/80 antibody (dilution 1:50; Serotec, Oxford, UK). The primary antibody was localized using the Vectastain ABC Elite Kit (Vector Laboratories) according to the manufacturer's instructions, followed by reaction with a 3,3′-diaminobenzidine substrate-chromogen solution kit (Vector Laboratories). The slides were counterstained with Harris hematoxylin. The numbers of F4/80-positive cells in each section were calculated by counting the number of positively stained cells in 10 fields per slide at a magnification of $\times 400$.

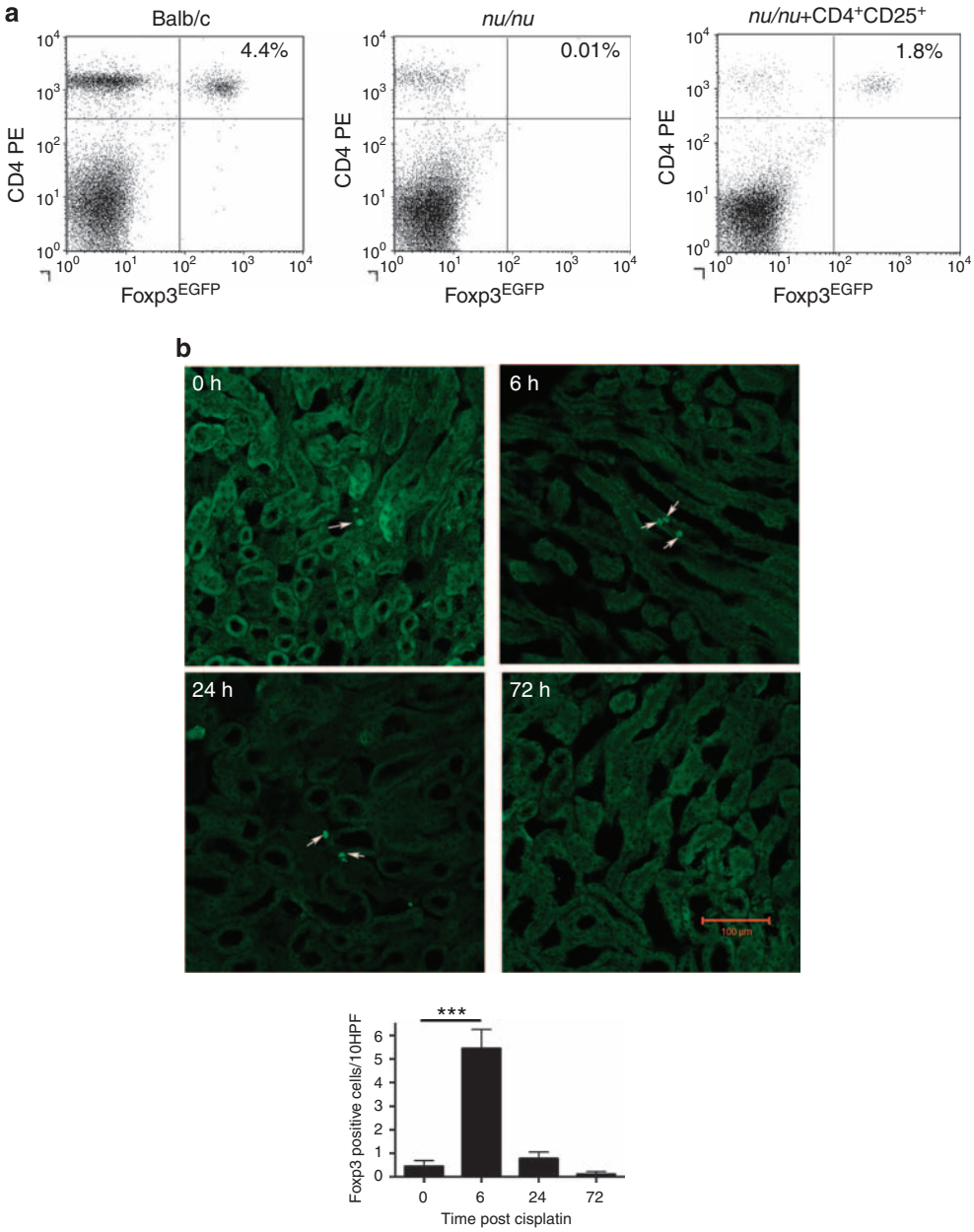

Figure 8 | Assessment of *Foxp3*^{EGFP} cell migration into the kidney. (**a**) 1×10^6 CD4$^+$CD25$^+$Treg cells from *Foxp3*^{EGFP} Balb/c mice were transferred to one group of *nu/nu* mice 10 days before cisplatin administration. Splenocytes were isolated from each mouse upon killing and then analyzed by FACS. *Foxp3*^{EGFP} Balb/c mice had 4.4 ± 0.06% CD4$^+$*Foxp3*^{EGFP}-positive cells; meanwhile, *nu/nu* mice had 0.01% CD4$^+$*Foxp3*^{EGFP}-positive cells in their spleen. At 10 days after an adoptive transfer of *Foxp3*^{EGFP} Balb/c CD4$^+$CD25$^+$ Treg cells, the average population of CD4$^+$*Foxp3*^{EGFP}-positive cells was reconstituted up to ***1.8 ± 0.06% (***$P < 0.001$ vs *nu/nu*). (**b**) *Foxp3*-positive cells in *nu/nu* mice kidneys were detected using a Zeiss LSM5 confocal microscope. The numbers of *Foxp3*-positive cells in each section were counted in 10 fields per slide (original magnification, × 200, ***$P < 0.001$ vs 0 h, $n = 3$). FACS, fluorescence-activated cell sorting; Treg, T regulatory cell.

Image analysis of renal *Foxp3*-positive cells

To confirm *Foxp3*^{EGFP} T-cell infiltration into the mouse kidney after cisplatin administration, CD4$^+$CD25$^+$ T cells were isolated from the spleens obtained from *Foxp3*^{EGFP} Balb/c mice. *nu/nu* Mice were reconstituted by tail intravenous injection with 1×10^6 CD4$^+$CD25$^+$-enriched T cells 10 days before cisplatin administration. CD4$^+$*Foxp3*^{EGFP} T cells from the spleen were confirmed by flow cytometry analysis, using PE-anti-mouse CD4 (BD Biosciences) (Figure 8a). Mice killed at different time points after cisplatin injection and kidneys were embedded in optimal cutting temperature compound (Sakura, Tokyo, Japan) and then cut into 20-μm sections. The renal tissues were permeabilized in 0.5% Triton X-100 and then incubated with a blocking

buffer. Subsequently, the samples were incubated with the anti-Foxp3 antibody (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The slides were exposed to fluorescein isothiocyanate-labeled secondary antibody (dilution 1:300; Santa Cruz Biotechnology). Fluorescent images were captured on a Zeiss LSM5 confocal microscope (Zeiss, Jena, Germany).

Depletion of $CD4^+CD25^+$ T cells *in vivo*

Anti-mouse CD25 rat IgG1 (anti-CD25; clone PC61) were generated in-house from hybridomas obtained from American Type Culture Collection (Manassas, VA, USA). A dose of 0.25 mg of anti-CD25 antibody was injected for 3 days before cisplatin administration. The efficacy of $CD4^+CD25^+$ Treg cell depletion was confirmed by flow cytometry analysis, using PE-anti-mouse CD25 and fluorescein isothiocyanate-anti-mouse CD4 (Figure 7a).

Statistical analysis

All data are presented as means ± s.e.m. Data were compared by an unpaired, two-tailed *t*-test for single comparisons or by ANOVA (analysis of variance) for multiple comparisons. Kaplan–Meier analysis was used for mouse survival analyses. Differences were considered to be significant if the *P*-value was <0.05.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This work was supported by the Korean Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (No. 2009-0063466). We thank Kyung Hee University for a Graduate Scholarship to H.L.

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